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14. ABSTRACT
Castration resistant prostate cancer (CRPC) is responsible for the overwhelming majority of prostate-cancer specific deaths. Although continued activation of the androgen receptor (AR) plays an important role in the development of castration resistance, AR-independent mechanisms represent an alternative source of biochemical signals that drive castration resistance. We have identified heightened expression of N-cadherin as a driving force in invasion, metastasis and castration resistance. Here we sought to identify biochemical signals that are regulated by N-cadherin in CRPC cells that can mediate the biologic effects of N-cadherin. In addition to activation of NF-kappa B reported in our first annual report, we have now identified the c-Jun N-terminal kinase (JNK) MAP kinase pathway as a signaling pathway that is activated in response to N-cadherin expression. We have generated inducible N-cadherin shRNA models to study the biochemical and cell biologic effects of N-cadherin. We have shown that N-cadherin activates JNK, which in turn phosphorylates the activator protein 1 (AP1) transcription factor c-Jun. Phosphorylated c-Jun then heterodimerizes with c-Fos and drives expression of proteins including Twist, that drive an epithelial-to-mesenchymal transition with heightened invasiveness. Heterodimers of c-Jun and c-Fos directly bind to the Twist promoter to drive Twist gene transcription. Pharmacologic inhibition of JNK reversed the effects of N-cadherin on invasion. Thus, in addition to NF-kappa B, the JNK pathway serves as another potential target for intervention. Ongoing work is focused on the biochemical pathways that link N-cadherin expression to JNK activation.

15. SUBJECT TERMS
Prostate cancer; N-cadherin; c-Jun N-terminal kinase; castration resistance.

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INTRODUCTION:

Metastatic castrate-resistant prostate cancer (CRPC) accounts for the vast majority of prostate cancer related deaths. Experimental and clinical evidence support the notion that reactivation of the androgen receptor (AR) seems to play a critical role in the emergence of the castrate-resistant state, and intensive investigative efforts have focused on the development of drugs that inhibit the androgen-AR signaling axis in unique and more effective ways.[1] Abiraterone acetate, an inhibitor of the CYP17 enzyme complex that mediates that rate limiting steps in androgen synthesis, irrespective of their source, including the tumor cell itself, received FDA approval for patients with metastatic, castration resistant prostate cancer who have already received docetaxel-based chemotherapy.[2] MDV3100, a potent androgen receptor antagonist, was recently reported to improve survival in post-chemotherapy metastatic CRPC. However, continued activation of AR signaling does not account for all cases of CRPC. Identification and targeting of the signaling pathways that promote AR-independent growth will be critical to advancing the treatment of CRPC.[3,4]

The theme of this proposal is centered around the role of increased N-cadherin expression in CRPC. We have previously credentialed N-cadherin as a viable target in CRPC, in that it is over-expressed in CRPC and its expression stimulates invasion, epithelial to mesenchymal transition (EMT), and tumorigenesis and promotes castration resistant growth.[5] To build on these findings, we have sought to identify pathways that may mediate and or function in conjunction with N-cadherin to promote prostate cancer growth. In our first year progress report, we emphasized the role of activation of the NF-κB pathway as a downstream mediator of N-cadherin. In addition, we identified AKT as an intermediate that delivers signals from N-cadherin to the NF-κB pathway.

JNK is a mitogen activated protein kinase (MAPK) that phosphorylates the Jun family of transcription factors, which homo- and hetero-dimerize to form transcriptional complexes known as activator protein 1 (AP1) transcription factors.[6] The literature has established an important role for the JNK/AP1 signaling pathway in oncogenesis.[7,8] For example, Jun is required Ras-induced transformation, and c-Jun is required for chemically induced hepatocellular carcinoma. In addition, the JNK and NF-κB share common upstream biochemical signals. Here, we describe the N-cadherin-dependent activation of the c-Jun N-terminus kinase (JNK) signaling pathway and provide evidence for its pathophysiologic role in prostate cancer growth.

BODY:

Task 1: Identify the biochemical signaling effectors of N-cadherin (Months 1-18; Rettig and Reiter).

a. Establish the specific IKK isoforms and NF-κB family members activated by N-cadherin (Months 1-3; Rettig)

   Previously reported.

b. Assess the interactions between N-cadherin and NF-κB, AKT and AR signaling . (Months 4-12; Rettig).

   Previously reported.

c. Determine the in vitro biologic effects of inhibiting N-cadherin along with downstream biochemical effectors (Months 13-18; Rettig and Reiter).

We have previously shown that N-cadherin expression enhances invasion and tumorigenesis. To further investigate N-cadherin biochemistry and cell biology, we developed inducible N-cadherin shRNA cell models. Here a lentiviral backbone that expresses N-cadherin specific shRNA under the regulation of a tetracycline-inducible promoter was stably introduced into LNCaP-C2 and PC3 cells. LNCaP-C2 cells stably express retrovirally introduced N-cadherin, whereas PC3 cells endogenously express N-cadherin. We employed two different N-cadherin targeting sequences, which yielded similar results. We show the results from one of the targeting sequences for each cell line. Of note, in generating the stable inducible cell lines, we isolated several clones and examined each clone for inducible knock-down of N-cadherin expression (Figure 1A), and selected one clone for detailed investigation (clone 4 for LNCaP-C2, and clone 3 for PC3). Silencing of N-cadherin expression by the addition of doxycycline markedly reduced the invasion of both LNCaP-C2 and PC3 cells in a Matrigel invasion assay (Figure 1B), a finding that indicts N-cadherin in the invasiveness of prostate cancer. Studies into the role of JNK signaling in invasiveness of prostate cancer cells is presented below.

Task 2: Establish the role of the extracellular and intracellular domains of N-cadherin on EMT, castrate-resistance, and downstream biochemical signaling. (Months 1-18; Reiter and Rettig).

a. Construct N-cadherin/E-cadherin chimeras and generate stable lines. (Months 1-6; Reiter).

Previously presented.

b. Assess the contribution of N-cadherin domains on *in vitro* and *in vivo* biologic endpoints, including invasion, metastasis, and androgen independence (Months 7-18; Reiter).

c.

Presented by initiating PI, Dr. Reiter.

d. Assess the contribution of N-cadherin domains on gene expression and downstream signal transduction (Months 7-18; Reiter and Rettig).

Effects of chimeras on PI3K/AKT signaling are presented by initiating PI, Dr. Reiter.

Task 3: Test efficacy of N-cad monoclonal antibodies in combination with inhibitors of biochemical signaling pathways downstream of N-cadherin in murine (Months 19-36; Reiter and Rettig).

a. Test the ability of N-cadherin antibodies alone and in combination with signaling inhibitors to inhibit tumorigenesis, induce regression and block metastasis in castrate mice (Months 19-36; Reiter).

Given the background described in the introduction, we postulated that JNK activity is dysregulated in prostate cancer cells that overexpress N-cadherin. Indeed, LNCaP-C2 cells, which ectopically express N-cadherin, manifest enhanced expression of phosphorylated (Ser 73) c-Jun (Figure 2A, left panels). Moreover, JNK activity, as directly measured by JNK *in vitro* kinase assays, was similarly hyperactivated in LNCaP-C2 cells (Figure 2A, right panels). To determine whether N-cadherin expression was biochemically linked to JNK activation, we silenced N-cadherin expression by exposing LNCaP-C2 and PC3 cells to doxycycline (1 μg/ml). Downregulation of N-cadherin led to reduced phospho-c-Jun and JNK activity (Figures 2B-C). Moreover, increasing concentrations of doxycycline led to a dose-dependent reduction in AP1 reporter activity (Figure 2D), a finding that further supports the link between N-cadherin and downstream JNK/AP1 activation.

b. Identify signaling pathways and biologic processes (e.g. angiogenesis, proliferation apoptosis) affected by above therapies (Months 19-36; Rettig and Reiter).

We next sought to determine mechanisms underlying the N-cadherin-dependent invasiveness and epithelial to mesenchymal transition. Using our tet-inducible cell models, we examined the expression of transcription factors, including Twist, Slug, Zeb1, and Zeb2, that regulate these biologic processes. Silencing N-cadherin expression with the addition of doxycycline, led to a reduction in Twist and Slug expression but no change in Zeb1 or Zeb2 levels (Figure 3A). Importantly, pharmacologic inhibition of JNK activity with SP600125 (hereafter termed JNKi) led to a dose-dependent reduction in Twist expression (Figure 3B). Inhibition of Twist expression by the JNKi was associated reduced invasiveness. In aggregate, these findings implicate AP1-dependent Twist expression in the N-cadherin regulated EMT and invasiveness of prostate cancer cells.

We next investigated the transcriptional regulation of Twist expression by AP1. To generate a map of putative AP1 binding sites in the *Twist* promoter, we employed M-Match, a publically available internet search tool for identifying transcription factor binding sites in DNA sequences. Analysis of the 2.5 kb region upstream of the *Twist* transcription start site identified numerous potential AP1 binding sites with varying homology to the consensus AP1 sequence (Figure 4A). Chromatin immunoprecipitation (ChIP) experiments identified a 350 bp region (-2,589 to –2,238 relative to the transcription start site; hereafter termed the “set 1” segment) containing four predicted AP1 binding sites to which c-Jun was recruited (Figure 4B). The specificity of the PCR signal was validated by the use of several controls: immunoprecipitation with non-specific IgG antibody, PCR amplification with water (no DNA), and PCR amplification with multiple sets of primers from other regions in the *Twist* 5’ UTR (Figure 4B). Because AP1 complexes are often composed of c-Jun and Fos family heterodimers, we predicted that c-Jun forms heterodimers with c-Fos on the AP1 DNA binding sites of the *Twist* promoter. Indeed, ChIP experiments confirmed that c-Fos was recruited to the set 1 region of the *Twist* promoter in a similar manner as c-Jun (Figure 4B).

KEY RESEARCH ACCOMPLISHMENTS:

- N-cadherin activates JNK/AP1.
- JNK/AP1mediates invasiveness and induction of EMT-regulated transcription factors (e.g. Twist) that are stimulated by N-cadherin.

- AP1 complexes composed of c-Jun and c-Fos heterodimers bind to the upstream regulatory elements in the 5' UTR of the *Twist* gene.

REPORTABLE OUTOMCES:

- Generation of inducible N-cadherin shRNA cell lines in LNCaP-C2 and PC3 background.

CONCLUSIONS:

N-cadherin expression induces invasiveness and EMT. This is mediated, at least in part, by downstream activation of the JNK/AP1 pathway. Specifically, N-cadherin expression induces activation of the MAP kinase, JNK, which in turn phosphorylates and activates c-Jun. Next, c-Jun forms a heterodimer with c-Fos to generate a competent AP1 transcriptional complex that induces the expression of a large cohort of genes, including *Twist*. These findings have important implications not only for the understanding of prostate cancer disease progression, but also for the development of a rational approach to drug development. For example, anti-N-cadherin antibodies could be used in conjunction with inhibitors of JNK. Moreover, inhibitors of NF-κB, including inhibitors of the kinase IKK, can be employed in combination therapy approaches with N-cadherin antibodies. Future directions will focus on co-targeting N-cadherin and other biochemical signaling pathways downstream of N-cadherin that cooperate with N-cadherin to drive invasion and tumorigenesis. As such, we will bring our model systems *in vivo* and exploit many of the compounds that are available to target these pathways. For example, JNK or IKK inhibitors can be used in conjunction with N-cadherin antibodies to effectively treat CRPC. In addition, we are identifying the biochemical pathways that link N-cadherin expression to JNK activation.

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APPENDICES:

None.

SUPPORTING DATA:

See attached figures.

Figure 1

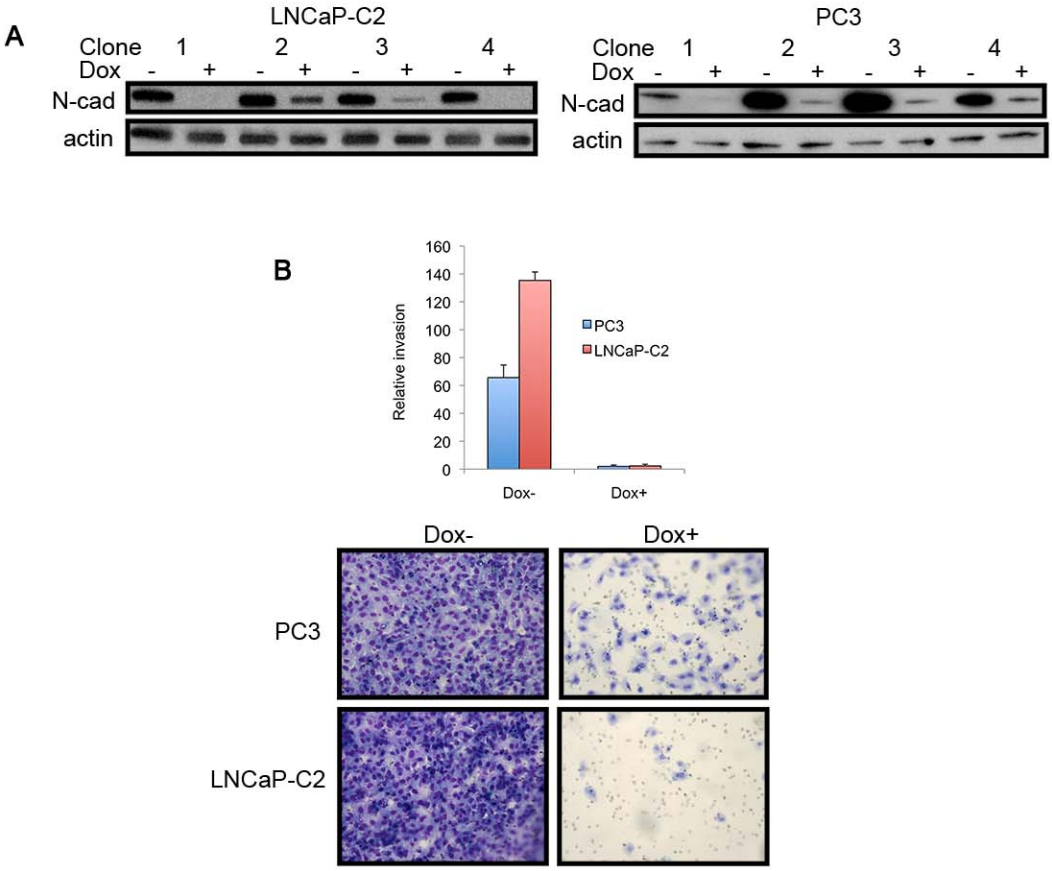

Figure 1. Inducible N-cadherin shRNA. A) LNCaP-C2 cells, which stably express N-cad, and PC3 cells, which endogenously express N-cad, were stably transduced with a lentivirus in which N-cad shRNA expression is tetracycine/oxycycline inducible. Shown are four clones for each cell line with N-cad expression depicted in the presence or absence of doxycycline (1 μg/ml). **B)** Invasion of LNCaP-C2 and PC3 cells is reduced upon induction of N-cad shRNA by doxycycline. The histogram at the top illustrates the marked difference in invasion in a Matrigel invasion assay; results are means of triplicates +/- s.d. Importantly, neither doxycycline nor N-cad shRNA affect the *in vitro* growth of cell lines during the time period of the assay, indicating that differences in invasion were *not* attributable to changes in rate of growth. Bottom panels are images of cells that have invaded into the Matrigel matrix (final magnification = 200X).

Figure 2

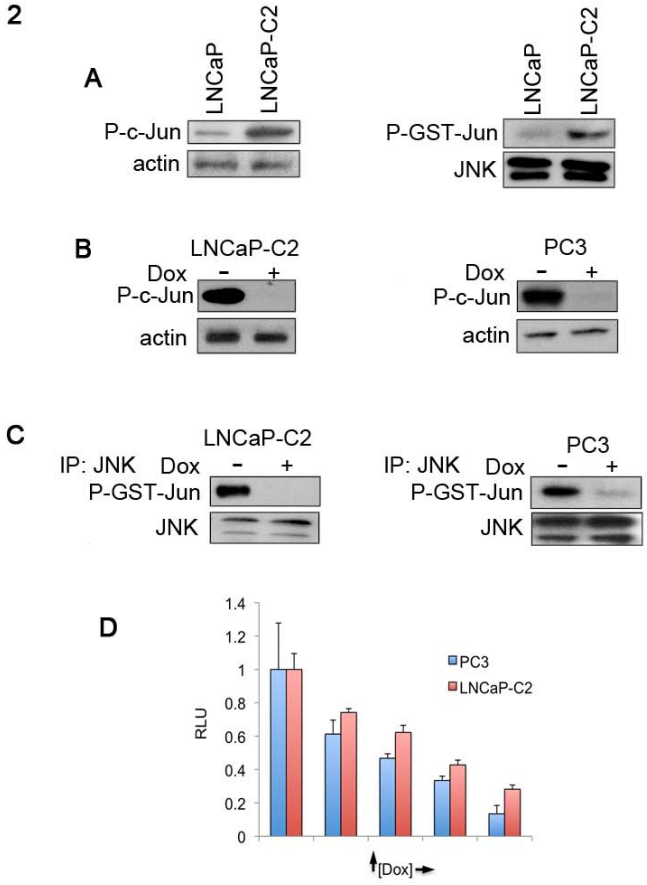

Figure 2. N-cadherin expression leads to activation of JNK/AP1. A) Increased phosphorylation of c-Jun (Ser 73) and JNK activity in LNCaP-C2 cells, which were engineered to stably express N-cad. Left panels are Western blots for the indicated proteins. Right panels represent a JNK *in vitro* kinase assay, in which JNK was immunoprecipitated; immunoprecipitates were subjected to a kinase assay, whereby phosphorylation of recombinant GST-c-Jun served as the read-out. **B)** N-cad silencing by inducing N-cad shRNA with doxycycline suppresses phosphorylation of c-Jun. **C)** Same as B) but read-out is JNK *in vitro* kinase assay. **D)** Increasing expression of N-cad shRNA by sequentially higher concentrations of doxycycline results in a dose-dependent reduction in AP1 reporter activity. Results are means of triplicates +/- s.d.

Figure 3

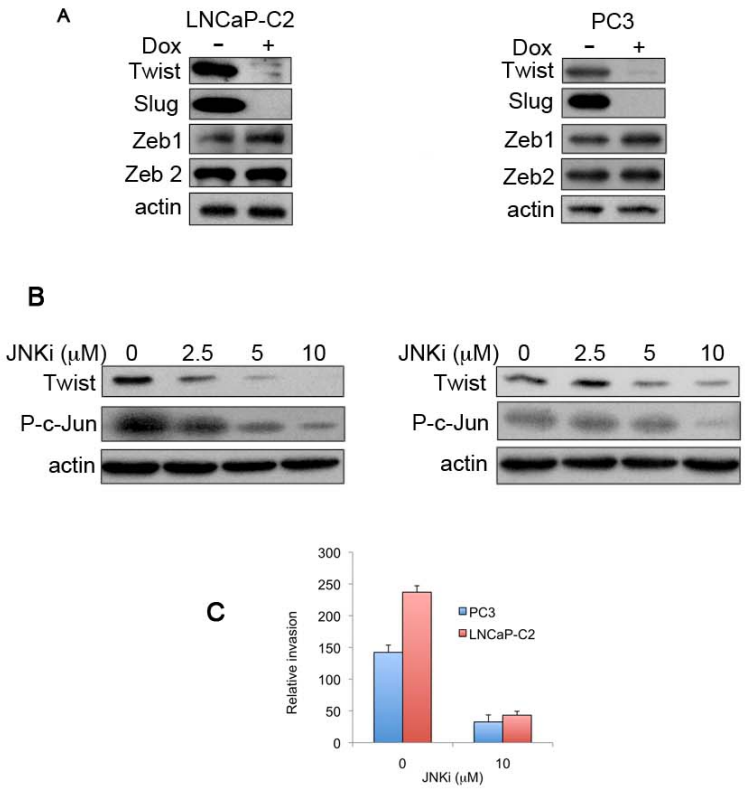

Figure 3. N-cadherin regulates Twist and Slug and invasion. A) Silencing N-cad expression through induction of N-cad shRNA with doxycycline reduced expression of Twist and Slug, but not Zeb1 or Zeb2. **B)** Twist expression is reduced by pharmacologic inhibition of JNK in a dose-dependent fashion by SP600125. **C)** SP600125 inhibits invasion in a Matrigel invasion assay. Results are means of triplicates +/- s.d.

Figure 4

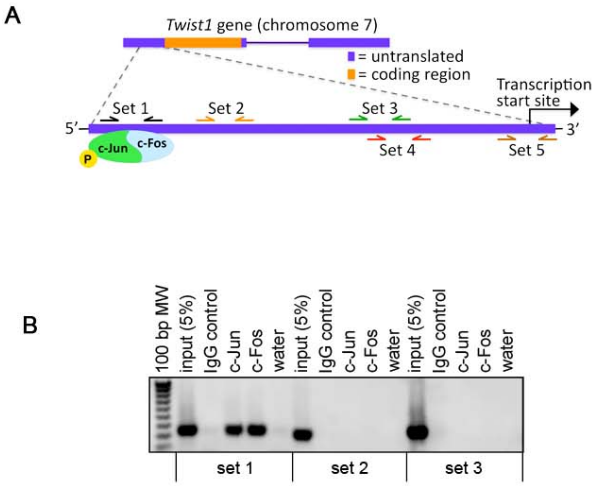

Figure 4. JNK/AP1-mediated regulation of *Twist* gene expression. A) Schematic figure of the *Twist* gene and promoter (modified from NCBI database). The *Twist* gene is located on chromosome 7p21. The ~2.5 kb upstream of the transcription start site was analyzed for putative AP1 binding sites with the aid of the M-Match search engine, and three PCR primers sets spanning predicted AP1 sites were generated for ChIP analysis. **B)** ChIP analysis of Twist promoter. DNA-protein extracts were immunoprecipitated with a c-Jun, c-Fos or IgG control antibody. Immunoprecipitates were subjected to PCR with five sets of primers covering various regions of the *Twist* promoter containing putative AP1 binding sites. PCR products are shown. PCR amplification of DNA not subjected to immunoprecipitation served as a positive control, and amplification of water was a negative control.